Exhibit 7

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Peptides Shorter Than a Minimal CTL Epitope May Have a Higher Binding Affinity Than the Epitope for the Class I K^k Molecule[1]

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A previously published K^k-specific motif was used to predict that an optimal K^k-restricted epitope within the nucleoprotein (NP) of influenza A/PR/8/34 virus corresponds to sequence SDYEGRLI (residues 50–57). Although this is the minimal epitope recognized by murine cytotoxic T lymphocytes (CTL), its binding affinity for the K^k molecule is increased following removal of either the N-terminal amino acid residue (S) or the N-terminal dipeptide (SD). A possible explanation for this unexpected result is that interactions between the C-terminus of the epitope and the K^k molecule contribute to the binding energy to a much greater extent than interactions between the N-terminus of the epitope and the K^k molecule.

Infection by influenza A virus is followed by a vigorous CTL response in mouse and man, which is directed predominantly against viral internal proteins (*1*) that undergo little genetic variation (*2*). The CTL response is thought to be important in limiting the spread of viral infection and in clearing viral disease (*3–5*), although experiments using transgenic mice deficient in β_2-microglobulin (β_2m) indicate that class I-restricted CTL are not essential for the clearance of influenza virus (*6, 7*). CTL generally recognize short peptides bound to syngeneic MHC class I molecules (*8*). These peptides are derived *in vivo* by cytoplasmic processing that might involve the proteasome (*9, 10*), although recent publications suggest that the proteasome may not be involved in the processing required for presentation of some epitopes (*11, 12*). The peptide epitopes are thought to be then transported via an ATP-dependent membrane transporter (*13*) into a pre-Golgi compartment, probably the endoplasmic reticulum (*14*), where they form a complex with MHC class I heavy chains and β_2m. The MHC class I–peptide complexes are then transported via the Golgi to the cell surface. Recent crystallographic evidence has suggested that hydrogen bonding between the two charged termini of a peptide epitope and "conserved" class I residues located at either end of the peptide binding cleft might be expected to contribute significantly to the binding affinity (*15–19*). Disruption of these hydrogen bonds might therefore be expected to significantly reduce the binding affinity (*20*).

Epitopes that are restricted by a particular MHC class I allele share a specific motif (*21, 22*), and a previously published K^k-specific motif (*23*) has recently been used to successfully predict the sequence of a minimal K^k-restricted epitope within the nonstructural NS1 protein of influenza A/PR/8/34 virus (*24*). This motif consists of a glutamic acid or, less frequently, an aspartic acid at position 2, and an isoleucine at the C terminus of a peptide eight or nine residues long (*23*). This K^k-specific motif was also used to predict a minimal K^k-restricted epitope within the NP of influenza A/PR/8/34 virus, corresponding to the sequence SDYEGRLI (residues 50–57) (*23*). Although this octapeptide had been shown to be much more efficient than a longer peptide, sequence SDYEGRLIQNSLTI (residues 50–63), at sensitizing target cells for lysis by an NP-specific murine CTL clone (*23*), evidence that it is the minimal or optimal epitope was not available.

To provide such evidence, the octapeptide SDYEGRLI and four other peptides homologous to this sequence but extended or truncated by one residue at either the amino or the carboxyl terminus were synthesized and HPLC purified. These peptides were tested (Fig. 1a) over a range of concentrations in the presence of L929 target cells (H-$2K^kD^k$) for recognition by an NP-specific K^k-restricted CTL clone JNP3, in a standard 5-hr ^{51}Cr-release assay (*25*). Peptides SDYEGRLI and LSDYEGRLI were equally efficient at sensitizing target cells to lysis, and each gave half-maximal lysis at a concentration of approximately 10^{-8} *M* (Fig. 1a). Peptide SDYEGRLIQ was recognized less efficiently (half-maximal lysis at approximately 5×10^{-7} *M*), and the shorter peptides DYEGRLI and SDYEGRL were not recognized over the range of concentrations tested (Fig. 1a). Thus peptide SDYEGRLI was the minimal NP epitope recognized by JNP3, but the optimal epitope

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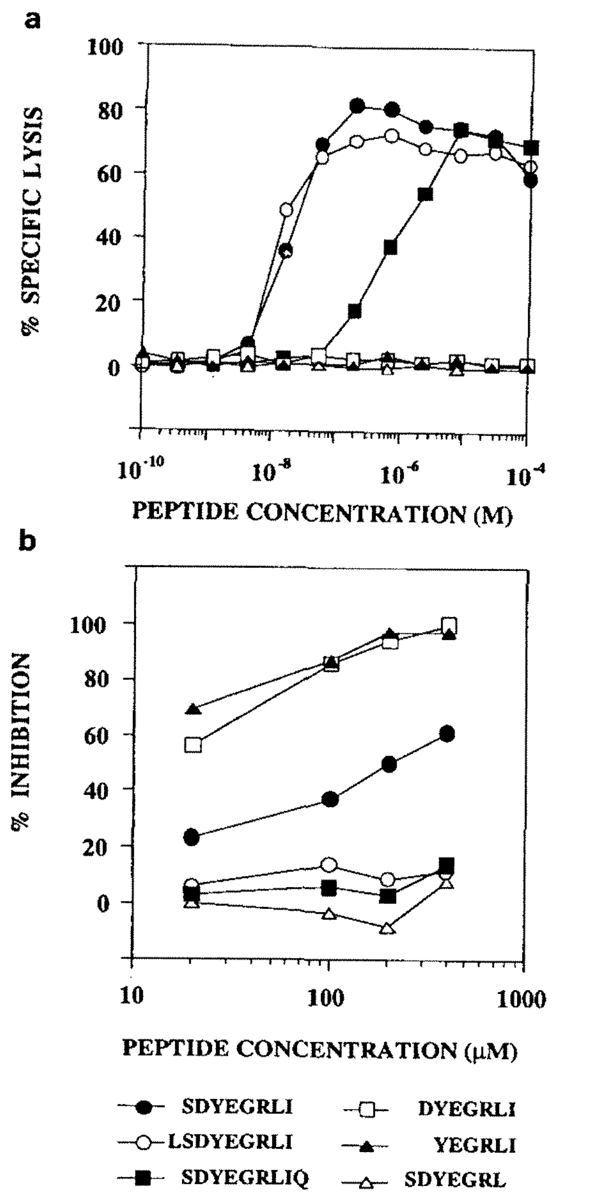

FIG. 1. (a) Recognition of NP peptides in a ^{51}Cr-release assay. ^{51}Cr-labeled L929 target cells were incubated with JNP3 effector cells at an effector:target ratio of 5:1 for 5 hr in the presence of varying concentrations of peptide as indicated. CTL clone JNP3 was derived by limiting dilution of a previously established NP-specific polyclonal CTL line (*23*) and was maintained as described elsewhere (*23*), using feeder cells that had been preincubated for 30 min with 5 μM peptide SDYEGRLI. (b) Functional competition assay to measure the relative binding affinities of the NP peptides. ^{51}Cr-labeled L929 target cells were incubated with HA8 effector cells at an effector:target ratio of 5:1 in the presence of 2 μM HA peptide epitope IEGGWTGMIDGW and varying concentrations of competing NP peptide as indicated, for 5 hours. % inhibition was calculated as 100 × ([% specific lysis no competition] − % specific lysis with competition])/[% specific lysis no competition].

could not be defined because the titration curves of peptides SDYEGRLI and LSDYEGRLI were indistinguishable (but see below).

The relative binding affinities of the above NP peptides for the K^k molecule were measured using an indirect functional competition assay (*24*). Briefly, L929 target cells were incubated with HA8 effector cells (a cloned hemagglutinin-specific CTL recognizing the epitope IEGGWTGMI (*23*)), in the presence of 2 μM hemagglutinin (HA) suboptimal epitope IEGGWTGMIDGW (*23*) and varying concentrations of competing NP peptide. The percentage of inhibition was calculated for the various peptide concentrations tested, and the results are plotted in Fig. 1b. The minimal epitope SDYEGRLI competed efficiently with the suboptimal HA epitope for binding to the K^k molecule as expected, and a 100-fold molar excess of peptide SDYEGRLI gave about 50% inhibition. By contrast, peptide SDYEGRL (lacking the C-terminal I of the K^k motif) did not inhibit recognition of the HA epitope over the range of concentrations tested. This was expected, firstly because the hydrogen bonds between the charged C-terminus of the peptide and the conserved K^k residues in the binding cleft would be disrupted, and secondly because the peptide lacked the C-terminal isoleucine anchor residue, thereby destabilizing the interaction with a binding pocket in the K^k molecule. The longer peptides LSDYEGRLI and SDYEGRLIQ did not significantly inhibit recognition of the sub-optimal HA epitope, thus indicating that these two NP peptides had low binding affinities for the K^k molecule relative to the minimal epitope (Fig. 1a). The naturally processed epitope would be expected to have a higher binding affinity than extended or truncated peptides for the restricting class I molecule. Therefore, peptide SDYEGRLI is strongly implicated as being the optimal epitope recognized by the CTL clone JNP3 because, compared with peptide LSDYEGRLI, it has a higher binding affinity for the K^k molecule.

Removal of the N-terminal serine residue from the optimal epitope SDYEGRLI significantly increased the binding affinity for the K^k molecule (Fig. 1b), and a 100-fold molar excess of peptide DYEGRLI gave 95% inhibition. This was unexpected, because removal of the N-terminal residue might be expected to disrupt the hydrogen bonds between the charged N-terminus of the peptide and "conserved" K^k residues within the binding cleft (*24*) and thus result in a significant decrease in the binding affinity, similar to that observed following the removal of the C-terminal residue (Fig. 1b). Because this peptide was not recognized by JNP3 (Fig. 1a), the N-terminal serine is likely to be a T cell receptor (TCR) contact residue. In order to examine the effect of a further deletion at the N-terminus, peptide YEGRLI (lacking the N-terminal serine and aspartic acid) was synthesized and HPLC purified. This peptide was expected to be a very poor inhibitor, because not only should the hydrogen bonds between the charged N-terminus and the K^k molecule be disrupted, but also the aspartic acid anchor residue (*23*) had been deleted. The relative binding affinity of this peptide was mea-

TABLE 1

Inhibition of Recognition of the Minimal NP Epitope SDYEGRLI by the Truncated Peptide YEGRLI

Concentration of YEGRLI (μM)	% Inhibition[a]
20	36
100	75
200	82
400	88

[a] Relative binding affinity of peptide YEGRLI was measured using an indirect competition assay (*24*). ^{51}Cr-labeled L929 target cells were incubated with NP-specific polyclonal CTL effector cells at an effector:target ratio of 5:1 in the presence of 10^{-7} *M* NP minimal epitope SDYEGRLI and various concentrations of competing peptide YEGRLI, as indicated. The specific lysis in the absence of competing peptide was 75%. The experiment was carried out three times, and this is an example of one set of results.

sured in a functional competition assay (see above), and unexpectedly peptide YEGRLI was as efficient as peptide DYEGRLI in that 100-fold molar excess gave 95% inhibition (Fig. 1b). Peptide YEGRLI was not recognized by JNP3 in a ^{51}Cr-release assay (Fig. 1a).

Because our assay for the binding of peptides to the MHC class I K^k molecule is an indirect competition assay, it is possible that peptide YEGRLI might not actually bind to the K^k molecule, but might instead bind to the antigen binding site on the TCR of CTL clone HA8, which would inhibit recognition of the HA epitope. This hypothesis was rejected, however, because peptide YEGRLI also efficiently inhibited recognition of the minimal peptide epitope SDYEGRLI by NP-specific polyclonal CTL (Table 1). This provided further evidence that peptide YEGRLI was binding specifically to the K^k molecule.

A possible explanation for the high binding affinity of peptide YEGRLI for the K^k molecule is that this peptide was binding to the K^k molecule in a highly extended conformation, with the glutamic acid residue at position 2 interacting as an anchor residue with the K^k molecule (analogous to the aspartic acid residue in the epitope SDYEGRLI). However, molecular modelling indicated that peptide YEGRLI cannot be extended sufficiently to permit this, nor allow the formation of hydrogen bonds between both the N and the C termini of the peptide and conserved K^k residues at each end of the binding cleft (Dr. P. Driscoll, personal communication). These conserved K^k residues are Y59, Y159 and Y171 which interact with the N terminus, and Y84, T143, K146, and W147 which interact with the C terminus of the epitope (*24*). This, together with the fact that deletion of the C-terminal isoleucine residue significantly decreased the binding affinity of the epitope for the K^k molecule, whereas removal of the first two N-terminal residues did not, suggests that the hydrogen bonds between the C terminus, but not the N terminus, of the epitope SDYEGRLI and the conserved K^k residues are maintained for peptide YEGRLI. An implication of these results is that the interactions between the isoleucine residue at the C terminus of the epitope SDYEGRLI contribute to the binding affinity for the K^k molecule to a much greater extent than the interactions between the N terminal residues and the K^k molecule. A possible explanation for the high binding affinity of peptide YEGRLI relative to that of the epitope is that residues YEGR of peptide YEGRLI might be making more favourable interactions than the corresponding residues in the epitope SDYEGRLI with the K^k molecule.

This is the first report in which truncation of a minimal epitope leads to a significant increase in the relative binding affinity for the restricting class I molecule. Such short peptides, if present in the ER, might inhibit or modulate the presentation of epitopes to specific CTL *in vivo*, thereby possibly interfering with the clearance of infected cells. However, the observations reported here might be specific for this particular NP epitope, and it remains to be seen if similar observations are true of other CTL epitopes.

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